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14. ABSTRACT
We previously showed that constitutive activation of the platelet-derived growth factor beta receptor (PDGFR) in mortal human fibroblasts (HDFs) by the bovine papillomavirus E5 or the v-Sis oncoprotein induces partial transformation of these cells. However, two weeks after they reach their peak density E5- and v-Sis-expressing HDFs secrete a small, hydrophilic peptide that induces massive apoptosis in an autocrine manner. Specifically, this peptide induces a type of caspase-independent, Bcl-2-resistant apoptosis by promoting mitochondrial dysfunction, which results in the release of the apoptotic mitochondrial protein AIF into the cytosol and its subsequent translocation to the nucleus. We hypothesize that as a negative feedback response to sustained PDGFR signaling, HDFs release a small, hydrophilic peptide that induces apoptosis by activating or sensitizing pro-apoptotic Bcl-2-related proteins such as Bax, which in turn promote mitochondrial dysfunction. The primary goal of this project is to identify this apoptotic peptide produced by partially transformed HDFs. Since this peptide can induce apoptosis of a number of different tumor cell lines including MCF-7 and MDA human breast carcinoma cells, once identified it could serve as the forerunner of a novel anti-breast cancer agent. Our evidence suggests that this peptide is <3 kDa, negatively charged, and pH sensitive. We recently obtained evidence to suggest that the peptide is a degradation product of an extracellular matrix protein. Moreover, we identified a peptide competitive inhibitor of the apoptotic peptide.

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INTRODUCTION:

We have been investigating the effect of sustained activation of growth factor receptor tyrosine kinases in mortal human fibroblasts (HDFs). Constitutive activation of the platelet-derived growth factor beta receptor (PDGFR) by the bovine papillomavirus E5 or the v-Sis oncoprotein induces partial transformation of HDFs [1]. However, two weeks after they reach their peak density HDFs partially transformed by E5 or v-Sis undergo massive apoptosis [2]. Our evidence suggests that these cells produce a small peptide that induces apoptosis in an autocrine manner. Specifically, this peptide induces a type of caspase-independent, Bcl-2-resistant apoptosis by promoting mitochondrial dysfunction, which results in the release of the apoptotic mitochondrial protein AIF into the cytosol and its subsequent translocation to the nucleus [2]. We hypothesize that as a negative feedback response to sustained PDGFR signaling, HDFs produce a peptide that induces apoptosis by activating or sensitizing pro-apoptotic Bcl-2-related proteins such as Bax, which in turn promote mitochondrial dysfunction. The primary goal of this project has been to identify the apoptotic peptide produced by partially transformed HDFs. Since this peptide can induce apoptosis of a number of different tumor cell lines including MCF-7 and MDA human breast carcinoma cells, once identified it could serve as the forerunner of a novel anti-breast cancer agent.

PROGRESS REPORT:

We attempted to identify the apoptotic peptide by searching for peptides that are present in apoptotic medium but not control medium. The overall strategy used is illustrated in Figure 1. Briefly, the low molecular weight medium fraction containing the apoptotic activity was isolated using centrifugal filtration devise with a molecular weight cut off size of 3 kilodaltons (kDa) and then concentrated three-fold. The low molecular weight fraction of medium from live control cells was isolated and processed in the same manner. The <3kDa medium fractions were then sent to Proteomic Research Services (Ann Arbor, MI) for MALDI/MS and LC/MS/MS analyses. MALDI/MS profiles and LC/MS/MS results were compared between the apoptotic and control samples.

Comparison of the MALDI/MS profiles revealed a peptide with a molecular mass of 1768 daltons and a mass:charge ratio of 885.01 present in the apoptotic medium but not in the control medium (Figure 2A). By subsequently committing LC/MS/MS data to *DeNovo* sequencing analysis the sequence of this peptide was determined to be: N-**(HA)PPPQPPRPQPPPQQ-C,** where the N-terminal amino acids are either HA or AH (Figure 2B). This peptide has significant homology to the N-terminus of fibrillin-2, a component of extracellular microfibrils in connective tissue. The 16-mer peptide having HA at its N-terminus (**HAPPPQPPRPQPPPQQ**) subsequently was chemically synthesized and tested for apoptotic activity on HDFs. Unfortunately, this peptide

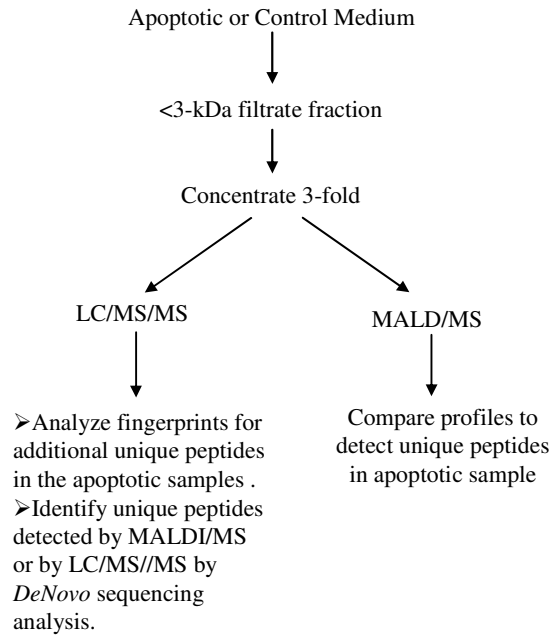
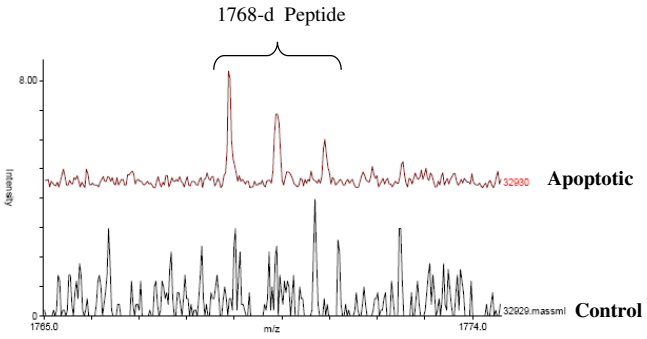


Figure 1. Strategy for identification of peptides present in apoptotic medium from E5-HDFs.

did not promote death of HDFs (data not shown), suggesting that it is not the apoptotic peptide produced by partially transformed HDFs. In the future we plan to test the apoptotic activity of the alternative peptide **AHPPPQPPRPQPPPQQ** (which has AH at its N-terminus).

Additional peptides differentially present in the apoptotic medium were detected by LC/MS/MS analysis and are listed in Table 1. Although LC/MS/MS analysis attempts to match a particular peptide to a known sequence in the data base, the matches for all but two of the unique peptides in the apoptotic medium received a low probability score and therefore were unlikely to be true matches. One of the two remaining peptides, a 1370.67 dalton peptide, matched a sequence within the pleckstrin homology domain of cytohesin-4, a guanine nucleotide exchange factor involved in vesicle formation and trafficking. This match received an intermediate probability score, and so, the likelihood of it being a true match is uncertain. The other peptide had a mass of 1656.98 daltons and matched a sequence contained within proMMP-2 (the zymogen form of matrix metalloproteinase-2 or gelatinase A), a secreted protease which binds to and degrades components of the extracellular matrix, particularly denatured collagen. This match received a relatively high probability score and is likely to be a true identity. The peptide specifically matched a sequence corresponding to the N-terminus of the pro-peptide of this enzyme. This particular region of MMP-2 binds intramolecularly to a fibronectin

A.



B.

1768-d peptide: **HAPPPQPPRPQPPPQQ**

Fibrillin-2: **QPPPPKPPRPQPPPQQ**

Figure 2. Identification of a small peptide specific to apoptotic medium from E5-HDFs. A. MALDI/MS profiles of low molecular weight fractions from Apoptotic and Control medium showing the presence of a 1768-dalton (d) peptide in the Apoptotic but not the Control medium. **B.** The sequence of the 1768-dalton peptide was determined by committing LC/MS/MS data for the Apoptotic sample to PEAKS software for Auto-*DeNovo* sequencing analysis followed by manual validation. The sequence of this peptide is shown in red in comparison with the highly homologous sequence of the N-terminus of Fibrillin-2.

Table 1. Peptides Specifically Detected in the <3kDa Fraction of Apoptotic Medium

Mr(daltons)	Predicted peptide sequence	Protein match
1768.00	(HA)PPPQPPRPQPPPQQ*	Fibrillin-2, N-terminus
1656.98	IIKFPGDVAPKTDKE**	proMMP-2, N-terminus
1370.67	ENLSVQKVDDPK***	Cytohesin-4 (pleckstrin homology, Sec7 and coiled/coil domains 4)
2042.06	Matches not significant	
1343.64	Matches not significant	
1307.67	Matches not significant	
1122.54	Matches not significant	

* identified by *DeNovo* sequencing

**received a high probability score of 50 and low expect value (the number of times we would expect an equal or higher score purely by chance) of 0.15, which is significant

***received an intermediate probability score of 30 and expect value of 45, which may or may not be significant.

type II (FN2) module adjacent to the catalytic domain and thereby induces a conformational change that inhibits the catalytic activity of the enzyme [3-5]. Cleavage of the N-terminal pro-peptide region by a cell surface-associated, membrane-type (MT)-MMP and other proteases relieves the allosteric inhibition, thereby activating the enzyme [3, 5]. Since MMP-2 is normally secreted by fibroblasts and its N-terminal pro-peptide domain is susceptible to multiple cleavage events, it stands to reason that a peptide derived from the N-terminus of MMP-2 would be present in the conditioned medium of HDFs.

Therefore, we decided to determine whether or not the pro-MMP-2 derived peptide played a role in apoptosis of the E5-HDFs. First, a synthetic form of the peptide was added to HDFs at various concentrations. However, the peptide displayed no apoptotic activity, even at a concentration of 400 μg/ml (data not shown). This suggests that this peptide is not the apoptotic peptide produced by partially transformed HDFs. Surprisingly, we found that prolonged treatment of HDFs with the pro-MMP-2-derived peptide inhibited apoptosis (data not shown). Unlike the E5 transformed HDFs, normal HDFs do not undergo apoptosis two weeks after confluence [2]. However, the normal HDFs eventually die within 50-60 days if cultured without a medium change. Apparently, the pro-MMP-2 peptide inhibited death of normal HDFs due to long-term culture. It is possible that the normal HDFs, like the E5-transformed HDFs, produce the apoptotic peptide, but do so after a much longer period in culture. If this is the case, the pro-MMP-2 peptide should inhibit the activity of the apoptotic peptide produced by the E5-HDFs. To test this possibility, normal HDFs were treated with the <3kDa apoptotic medium fraction in the absence or presence of increasing concentrations of the pro-MMP-2 peptide. Several days later the viability of the cells was assessed as shown in Figure 3. As expected and shown previously [2], the <3kDa apoptotic fraction efficiently killed the cells, reducing the viability to less than 2%. However, the presence of the pro-MMP-2-derived peptide reduced the ability of the <3kDa medium fraction to kill the cells, enhancing the viability of the cells in a dose-dependent manner. A control peptide (the fibrillin-2-like peptide also found in the conditioned medium) when used at a relatively high concentration only minimally reduced the apoptotic activity of the <3kDa medium fraction. Therefore, the pro-MMP-2 peptide specifically and dose-dependently reduced the death-inducing activity of the apoptotic peptide.

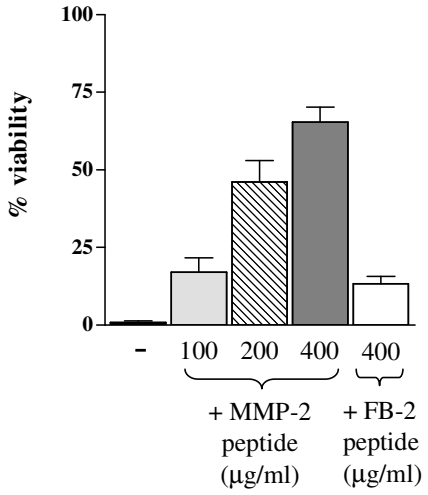


Figure 3. The MMP-2-derived peptide inhibits the apoptotic activity in the conditioned medium of E5-HDFs. The <3kDa fraction from the apoptotic medium was added to normal HDFs in the absence (-) or presence of the MMP-2-derived peptide or the Fibrillin-like (FB-2) peptide at the indicated concentrations. (The sequence of both peptides is shown in Table 1). Several days later the cells were trypsinized and stained with trypan blue and counted for determination of cell viability. Viability was determined as the percent of unstained live cells versus the total number of cells. Mean values with standard error from cells treated in triplicate are shown.

The pro-MMP-2-derived peptide could inhibit the apoptotic peptide by directly interacting with it or by competitive inhibition, i.e., binding to but not affecting the target of the apoptotic peptide. We believe the latter possibility to be the case, since pre-incubating the apoptotic medium fraction with the lowest concentration of the pro-MMP-2-derived peptide did not enhance its inhibitory effect on apoptosis (data not shown). Nonetheless, in either case, we may be able to identify the apoptotic peptide by identifying proteins that interact with the pro-MMP-2 peptide. Even if the pro-MMP-2 peptide is

a competitive inhibitor, identifying proteins that interact with it may allow us to identify the target of the apoptotic peptide. Once identified, the target then could serve as an affinity ligand for subsequent purification of the apoptotic peptide.

If the pro-MMP-2-derived peptide is a competitive inhibitor and binds to the same target as the apoptotic peptide, it stands to reason that the two peptides may be similar with respect to their amino acid sequence. After performing a BLAST search, we found that amino acids 843-852 of collagen type 18 shared 80% sequence identity with the pro-MMP-2 derived peptide (Figure 4). Collagen 18, a proteoglycan collagen, is an extracellular matrix component of basement membranes. The anti-angiogenic peptide endostatin is a C-terminal fragment of collagen 18 generated after cleavage by extracellular proteases such as MMPs and cathepsin-L [6]. Although the region homologous to the pro-MMP-2 peptide is not in the endostatin domain of collagen 18, it would be reasonable to assume that this collagen may be proteolytically cleaved at other sites to generate other bioactive peptides, including an apoptotic peptide. In fact, the region of homology with the pro-MMP-2 peptide is flanked by two motifs resembling MMP cleavage sites (Figure 4)[7]. Consistent with the notion that the apoptotic peptide is derived from an MMP cleavage product of collagen 18, we recently found that MMP's are required for generation of the apoptotic peptide, as both pan-MMP and MMP-2-specific inhibitors inhibited production of the peptide by E5-HDFs (Table 2). Moreover, a peptide corresponding to residues 839-852 of collagen type 18, which includes the pro-MMP-2- homologous domain, has a predicted molecular weight of 1370.68 daltons, which is nearly the same mass as one of the peptides differentially produced by the E5-HDFs (Table 1). Therefore, we plan to test the apoptotic activity of a synthetic peptide corresponding to residues 839-852 of collagen type 18 (Figure 4, sequence in red) because this peptide shares homology with a competitive inhibitor peptide (the pro-MMP-2 peptide), is the same mass as one of the unique peptides detected in the apoptotic medium, and could be generated by MMPs, which appear to play role in production of the apoptotic peptide.

Figure 4. Sequence alignment of the pro-MMP-2-derived peptide and a region in collagen type 18. Identical amino acids are indicated by lines between the sequences. Underlined sequences in collagen 18 resemble MMP cleavage sites and arrows indicate site of cleavage. The sequence in red comprises a peptide that is 1370.68 daltons, which is the same size of one of the peptides detected exclusively in the apoptotic medium. Numbers at the top refer to the position of amino acids in collagen 18.

We also obtained further evidence that the apoptotic peptide has an overall negative charge in its active form. Pre-incubation of the <3 kDa apoptotic medium fraction with a positively charged nylon membrane substantially reduced the apoptotic activity of the medium fraction, while pre-incubation with nitrocellulose had no effect on the apoptotic activity (Figure 5). This suggests that the apoptotic peptide has an overall negative charge and was depleted from the medium by binding to the positively charged membrane in an electrostatic manner. This is consistent with our previous observation that the apoptotic peptide is not active at a pH below 7.5, suggesting that the isoelectric point (pI) of the active form is lower than 7.5. The fact that the pI of the collagen 18-derived peptide (indicated in red in Figure 4) is 5.96, lends further support for it being a candidate for the apoptotic peptide.

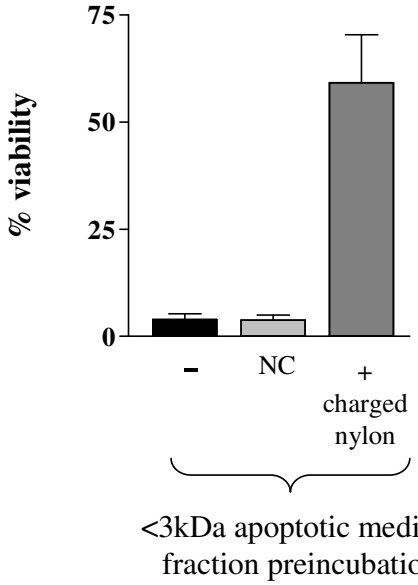


Figure 5. Depletion of the apoptotic activity from the <3kDa conditioned medium fraction with a positively charged nylon membrane. The < 3kDa apoptotic medium fraction was either untreated (-) or pre-incubated with a nitrocellulose (NC) or positively charged nylon membrane for several hours. The untreated or treated apoptotic medium was added to HDFs in duplicate by medium replacement. Several days later the cells were assessed for viability by trypan blue dye exclusion assay. Values shown are the mean percent viability with standard error.

Table 2. Effect of Various Inhibitors on Apoptosis of E5 HDFs

Inhibitor	Target enzyme	Inhibition* of apoptosis of E5-HDFs
LY294002	PI 3'-kinase	+
Wortmannin	PI 3'-kinase	+
SP600125	JNK	+
Y27362	Rho kinase	delayed
PPI	Src	+
AG1296	PDGF receptor	+
PD98059	MEK-1/2	-
U73122	PLC–γ	-
APHS	Cox2	-
Cyclosporin A	Cyclophillin A	-
MMP inhibitor III	multiple MMPs	delayed
MMP-2 inhibitor	MMP-2	delayed
Furin inhibitor	Furin	-
15dPGJ2	PPARγ; others	+
CAY10410	PPARγ	-

"+" or "delayed" indicates that an inhibitory effect was observed only when the inhibitor was added prior to but not after confluence, suggesting that inhibition occurred at the level of production of the apoptotic peptide.

In any case, the property of binding to the positively charged membrane can be exploited for purposes of purification of the apoptotic peptide. Specifically, peptides from control medium and apoptotic medium will be incubated with the positively charged membrane. Bound peptides will be eluted from the membrane, and eluates will be subjected to LC/MS/MS analysis. Bound peptides present in the apoptotic medium but not the control medium would be reasonable candidates for the apoptotic peptide.

Finally, we tested the ability of various pharmacological inhibitors to prevent production of the apoptotic peptide. Briefly, E5-HDFs were treated with the inhibitors listed in Table 2 prior to confluence and then monitored for signs of apoptosis two weeks later. As indicated in Table 2, we observed that several kinase inhibitors, including those for the PDGF receptor, PI 3-kinase, Src, and JNK completely inhibited apoptosis, but only if added prior to confluence. This suggests that E5-induced activation of the PDGF receptor and downstream activation of certain of this receptor's signaling kinases are required for production of the peptide. Furthermore, as mentioned above, we also found that MMP inhibitors delayed apoptosis, but only if added prior to confluence. This suggests that MMPs are also involved in production of the peptide. On the other hand, inhibition of Furin, another protease involved in post-translational processing of membrane and secreted proteins, did not inhibit

apoptosis of E5-HDFs, suggesting that Furin-mediated proteolysis does not play a role in generation of the apoptotic peptide. Therefore, we speculate that activation of certain PDGF receptor signaling pathways results in activation of MMPs, which in turn promote degradation of extracellular matrix proteins leading to production of the apoptotic peptide. We believe that the apoptotic peptide is a bioactive degradation product of an extracellular matrix protein such as collagen.

We also found that 15d-PGJ_2, a naturally occurring metabolite of prostaglandin J_2, inhibits production of the apoptotic peptide at a concentration of 12.5 μM. E5-transformed HDFs treated with 15d-PGJ_2 prior to but not after confluence were protected from apoptosis. This suggests that 15d-PGJ_2 inhibits the production but not the activity of the apoptotic peptide. 15d-PGJ_2 is a potent agonist for the nuclear receptor PPARγ [8] but also can act in a PPARγ-independent manner by forming adducts with free sulfhydryl moieties of cysteine residues on cellular proteins [9]. Therefore, we tested whether or not CAY10410, a 15d-PGJ_2 analog that is a PPARγ agonist but cannot form adducts with proteins, can inhibit apoptosis of the E5-transformed HDFs. We found that CAY10410 could not prevent apoptosis of these cells, suggesting that 15d-PGJ_2 prevents production of the apoptotic peptide in a PPARγ-independent manner, most likely by forming an adduct with a protein involved in the proteolytic pathway required for production of the peptide. For example, 15d-PGJ_2 could interact with the sulfhydryl of the critical cysteine residue in the catalytic cleft of an MMP and thereby inhibit its activity.

Summary of key Accomplishments:

- MALDI/MS and LC/MS/MS analyses were able to detect peptides differentially present in apoptotic medium versus control medium. Two of these peptides corresponded to the N-terminus of secreted proteins. One appeared to be derived from the extracellular matrix protein, fibrillin-2, while the other was derived from the pro-form of MMP-2. Other peptides were detected but could not be positively identified.
- Although neither the fibrillin-2-like peptide nor the pro-MMP-2 peptide proved to be apoptotic, the proMMP-2 peptide inhibited apoptosis induced by our apoptotic medium fraction. Further evidence suggested that the pro-MMP-2 peptide is a competitive inhibitor of the apoptotic peptide. The pro-MMP-2 peptide could be used to identify the apoptotic peptide's target protein, which then could be used to purify the apoptotic peptide for subsequent identification.
- It is possible that the apoptotic peptide shares sequence similarity with its competitive inhibitor, the pro-MMP-2 peptide. A BLAST search revealed that a region in collagen 18 shares 80% homology with the pro-MMP-2 peptide and therefore could be a candidate for the apoptotic peptide. This region in collagen 18 is flanked by two putative MMP cleavage sites, and thus, a peptide homologous to the pro-MMP-2 peptide could be generated by MMP-cleavage of collagen 18 in the extracellular milieu.
- Our evidence indicated that MMP's are required for generation of the apoptotic peptide, suggesting this peptide is a proteolytic degradation product of an extracellular matrix protein such as collagen. This is consistent with the notion that the apoptotic peptide is derived from a region in collagen 18 that is homologous to the proMMP-2 peptide.
- The apoptotic peptide appeared to bind to a positively charged nylon membrane, suggesting that it has an overall negative charge in its active form. The ability of the peptide to bind to this membrane will be exploited to more adequately purify the peptide for subsequent identification.

- A metabolite of prostaglandin J2, 15d-PGJ_2, inhibited production of the peptide perhaps by forming an adduct with and inhibiting an extracellular protease that is required for production of the peptide.

CONCLUSIONS:

Although we have not yet identified the apoptotic peptide, the new data obtained in the past year has provided additional clues to its identity. These data suggest that the apoptotic peptide is derived from MMP-mediated degradation of an extracellular matrix or transmembrane protein. One possibility is that the apoptotic peptide is a 1370.68 dalton peptide corresponding to residues 839-852 of collagen 18, an MMP substrate. In support of this hypothesis, this collagen 18-derived peptide shares sequence homology with a competitive inhibitor peptide identified in the apoptotic medium and is the same size as another peptide detected in the apoptotic medium. To test this hypothesis we will assess the apoptotic activity of a synthetic form of the collagen-18 derived peptide. In the potential event that this hypothesis is incorrect, we now have some tools to more adequately purify the apoptotic peptide for subsequent identification. First, since the peptide binds to a positively-charged nylon membrane, we can use this membrane as a means of purifying the peptide. Second, we can use the competitive inhibitor peptide that we identified as a means of identifying the target protein of the apoptotic peptide. Once the target protein is identified, we can use it as an affinity ligand for purification of the apoptotic peptide. In addition, the identity of the target protein should reveal new clues into the mechanism by which the apoptotic peptide induces cell death. Overall, we are optimistic about identifying this peptide in the near future.

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